ELSEVIER

European Journal of Pharmacology 361 (1998) 101–108

ejp

Sulfatide reduces leucocyte accumulation and reverts vascular failure in splanchnic artery occlusion shock

Francesco Squadrito [a], Domenica Altavilla [a,*], Giovanni Squadrito [b], Giuseppe M. Campo [a], Mariarita Arlotta [a], Cristina Quartarone [b], Letteria Minutoli [a], Marcella Ferlito [a], Antonino Saitta [b], Achille P. Caputi [a]

[a] *Institute of Pharmacology, School of Medicine, University of Messina, Piazza XX Settembre, 4 98122 Messina, Italy*
[b] *Department of Internal Medicine, School of Medicine, University of Messina, Piazza XX Settembre, 4 98122 Messina, Italy*

Received 7 August 1998; revised 21 September 1998; accepted 25 September 1998

Abstract

Selectin-mediated leucocyte accumulation is implicated in the pathogenesis of splanchnic artery occlusion. Sulfatide is recognized by P-selectin and blocks this adhesion molecule. We investigated the effects of sulfatide in rats subjected to splanchnic artery occlusion shock. Anaesthetized rats, subjected to total occlusion of the superior mesenteric artery and the coeliac trunk for 45 min developed severe shock resulting in death within 85–90 min after the release of occlusion. Sham operated animals were used as controls. Splanchnic artery occlusion shocked rats had marked hypotension, enhanced levels of tumor necrosis factor-α (TNF-α) in serum and macrophages, leucopenia and increased ileal leucocyte accumulation, studied by the means of myeloperoxidase activity. Furthermore, aortae from shocked rats showed marked hyporeactivity to phenylephrine (1 nM–10 μM), reduced responsiveness to acetylcholine (10 nM–10 μM) and an increased staining for P-selectin in the vasculature. In vivo administration of sulfatide (10 mg/kg, i.v., 5 min after occlusion of the splanchnic arteries) increased survival rate (90%, 4 h after splanchnic artery occlusion shock), enhanced mean arterial blood pressure, reduced serum TNF-α (37 ± 11 U/ml vs. 398 ± 18 U/ml), ameliorated leucopenia and reduced ileal myeloproxidase activity (1.2 ± 0.4 U/g tissue vs. 8.2 ± 0.8 U/g tissue). Aortae from splanchnic artery occlusion shocked rats treated with sulfatide exhibited a greater contractile response to phenylephrine and improved responsiveness to acetylcholine. Moreover sulfatide-treated rats showed a reduced staining for P-selectin in the aorta and in the superior mesenteric artery. Finally, passive immunization with specific monoclonal antibodies raised against P-selectin significantly protected from the lethality induced by splanchnic artery occlusion shock. Our results suggest that sulfatide protects against splanchnic artery occlusion shock.

Keywords: Sulfatide; Ischemia-reperfusion injury; Selectin

1. Introduction

In recent years many of the molecular structures on the cell surface that mediate adherence have been identified. In general, adherence seems to occur in two steps. First, neutrophils are bound only transiently to the endothelial cells, a process which mainly involves the P- and E-selectins (Bevilacqua et al., 1987; Patel et al., 1991) on endothelial cells and L-selectin on neutrophils (Key et al., 1991).

This transient adherence or so-called 'rolling' is then replaced by definitive adherence that is mediated by intracellular adhesion molecule 1 (ICAM-1) on endothelial cells (Smith et al., 1989) and integrins (CD18/11a, CD18/11b, CD18/11c) on neutrophils (Welbourn et al., 1992).

It has been shown that blocking of neutrophil adhesion molecules significantly decreases ischaemia-reperfusion injury (Romson et al., 1983; Simpson et al., 1988; Welbourn et al., 1992). Because neutrophil rolling is the first step of adhesion, it seems, therefore, to be most reasonable to block one of the selectin molecules.

Selectins located on endothelial cells (P- and E-selectins) exhibit organ-specific differences in their pathophysiological relevance (Mulligan et al., 1993) while L-selectin, which is located on neutrophils might represent a useful

* Corresponding author. Tel.: +39-90-712533; Fax: +39-90-661029

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00705-5

target for an anti-adhesion strategy (Arfors et al., 1987). It has been shown that sulfatide, sulfated galactosylceramide, recognizes and blocks P-selectin (Needham and Schnaar, 1993).

Occlusion of the major splanchnic arteries followed by reperfusion in anaesthetized rats results in an irreversible circulatory failure and shock (splanchnic artery occlusion shock; SAO shock) and leads to the death of the animals within 80–90 min after the release of occlusion (Squadrito et al., 1994a).

Previous findings from our laboratory have shown that endothelium–leucocyte interactions play an important role in the pathogenesis of splanchnic artery occlusion shock. Neutrophil depletion induced by the administration of vinblastine results in an increased resistance of rats to the pathological sequelae of intestinal ischaemia-reperfusion injury (Canale et al., 1993). Furthermore, it has been shown that the mechanisms supporting leucocyte rolling and accumulation in the endothelium (which are mediated by selectins and intercellular adhesion molecule 1, respectively), are operative in this model of experimental shock (Squadrito et al., 1992; Squadrito et al., 1994b; Altavilla et al., 1995).

In the light of these findings, we studied whether sulfatide can limit leucocyte–endothelial interactions thus protecting against the pathological sequence associated with the occlusion and reperfusion of the splanchnic region. Our data suggest that either sulfatide or a monoclonal antibody raised against P-selectin protect against splanchnic artery occlusion shock.

2. Methods

2.1. Animal preparation

Male Sprague–Dawley rats weighing 200–250 g were permitted access to food and water ad libitum. The rats were anaesthetized with urethane (1.3 g/kg, i.p.). After midline laparotomy, the coeliac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed 0.9% NaCl solution. Rats were given heparin (1000 U/kg, i.v.) and were observed during a 30-min stabilization period prior to either splanchnic ischaemia or sham ischaemia. Splanchnic artery ischaemia-reperfusion injury was induced by clamping both the superior mesenteric artery and the coeliac trunk so as to produce total occlusion of these arteries for 45 min. The clamps were then removed. Following reperfusion the rats were observed for 4 h. Sham-operated rats were subjected to the same surgical procedures as shocked rats except the arteries were not occluded.

2.2. Survival evaluation and arterial blood pressure monitoring

A first group of animals was used to study survival and arterial blood pressure. Five minutes following occlusion of the splanchnic arteries, treated rats received sulfatide (2.5, 5 and 10 mg/kg, i.v.), vehicle (1 ml/kg, i.v.) or monoclonal antibodies raised against P-selectin (P-selectin mAb 2 mg /kg, i.v.). Survival was evaluated for 4 h after the onset of reperfusion and expressed either as survival rate or survival time. A group of animals was also implanted with cannulae (PE 50) into the left common carotid artery, as described elsewhere (Caputi et al., 1980). The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was displayed on a polygraph. Arterial blood pressure is reported as mean arterial pressure in mm Hg. Rats were subjected to the same experimental protocol as described above.

2.3. Biological assay for tumor necrosis factor-α activity

A third group of animals was used to measure tumor necrosis factor (TNF-α), myeloperoxidase activity, leucocyte count, vascular reactivity and P-selectin expression on the vascular endothelium.

Killing of L929 mouse tumour cells was used to measure TNF-α levels in serum and in peritoneal macrophage supernatants on the basis of a standard microelisa assay (Ruff and Gifford, 1980). L929 cells in RPMI 1640 medium containing 5% fetal calf serum were seeded at 3×10^4 cells/well in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial 1:2 dilutions of serum (drawn 70 min following the onset of reperfusion) and supernatants of peritoneal macrophages (we measured only the spontaneous release of the cytokine by the macrophages), harvested at the same time as the serum using a previously described method (Altavilla et al., 1989), were made in the above-described medium containing 1.0 μg of actinomycin D/ml, and 100-μl vols. of each dilution were added to the wells. One TNF-α unit was defined as the amount causing 50% cytotoxicity. The TNF-α content in the sample was calculated by comparison with a calibration curve obtained with recombinant murine TNF-α (Nuclear Laser Medicine, Milan, Italy). To test whether the cytotoxicity was due to the presence of TNF-α or to other factor(s), we preincubated our samples for 2 h at 37°C with an excess of rabbit anti-recombinant murine TNF-α polyclonal antibodies (Nuclear Laser Medicine, Milan, Italy), or with control rabbit serum. Our results showed that cytotoxicity against L929 cells was completely neutralized by rabbit anti-recombinant TNF-α polyclonal antibodies, but not by control rabbit serum.

2.4. Myeloperoxidase activity and leucocyte count

Leucocyte accumulation was investigated by measuring the activity of myeloperoxidase in intestinal mucosa, as previously reported (Mullane et al., 1985). The samples of intestinal mucosa were obtained at 0 min before occlusion of the splanchnic arteries and at 70 min following the onset of reperfusion. The samples were first homogenized in a solution containing 20 mM of potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 U/ml of a protease inhibitor (aprotinin) in proportions of 1:10 (w:v) and then centrifuged for 30 min at 20,000 × *g* at 4°C. The supernatant of each sample was then discarded and the pellet was immediately frozen on dry ice. The samples were kept at 0°C for 14 h before sonication. After thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma, St. Louis, MO, USA) dissolved in 50 mM potassium phosphate buffer (pH 6) containing 30 U/ml of protease inhibitor. Each sample was then sonicated (intensity 2) for 1 min at a temperature of 4°C. After sonication the samples were allowed to chill on ice for approximately 30 min, and then they were centrifuged for 30 min at 40,000 × *g* at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma) and 0.001% H_2O_2, and the rate of change in absorbance was measured at 405 nm in a microtitre plate reader. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 25°C and is expressed in units g weight (U/g of tissue). Tail vein blood samples for the leucocyte count were taken at 0 min before initiating reperfusion and at 70 min after the onset of reperfusion. The number of white blood cells ($WBC \times 10^3 \times mm^3$) is reported as mean ± S.E.M.

2.5. Isolated aortic rings

Thoracic aortae were removed 70 min after reperfusion and placed in cold Krebs' solution of the following composition (nM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and glucose, 11.7: then the aortae were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings, the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick. Rings were then placed under 1 g of tension in an organ bath containing 10 ml of Krebs' solution at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, ITALY). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs' solution at 15 to 20 min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (100 nM). When the contraction was stable, the functional integrity of the endothelium was assessed by determining the relaxant response to acetylcholine (100 nM). The tissue was then washed occasionally for 30 min. Endothelium-dependent relaxation was evaluated with cumulative concentrations of acetylcholine (10 nM–1 μM) in aortic rings precontracted with phenylephrine (100 nM). Relaxation of the rings was calculated as the percent decrease in contractile force. Concentration–response curves were obtained with cumulative concentrations of phenylephrine (1 nM–10 μM) added to intact or endothelium-denuded aortic rings.

2.6. Immunohistochemistry

P-selectin expression was studied in thoracic aortae and in superior mesenteric arteries collected 70 min following release of the occlusion. Immunohistochemical evaluation was accomplished by staining 5-μm thick cryostat sections according to the avidin–biotin–peroxidase complex procedure (Hsu et al., 1981). An average of seven sections per immunohistochemical stain were cut from each sample, airdried for 30 min and then fixed in cold acetone for 10 min. Endogenous peroxidases were blocked with horse serum for 15 min at room temperature prior to incubation with primary antibodies. The monoclonal antibodies used were raised against mouse P-selectin (clone: RB40.34) and were obtained from Pharmingen (San Diego, CA USA). A monoclonal mouse immunoglobulin G_1 (IgG_1) antibody was used for controls. Biotinylated, species-specific sec-

Table 1
Effect of sulfatide and P-selectin mAb on survival in splanchnic artery occlusion (SAO) shocked rats

Treatment	Survival time (min)	Surviving animals	Survival rate (%)
Sham + vehicle (1 ml/kg)	> 240	10/10	100
Sham + sulfatide (10 mg/kg)	> 240	10/10	100
SAO + vehicle (1 ml/kg)	80 ± 10[a]	0/10[a]	0
SAO + P-selectin mAb (2 mg/kg)	229 ± 9[c]	9/10[c]	90
SAO + sulfatide (2.5 mg/kg)	100 ± 5[a]	0/100[a]	0
SAO + sulfatide (5 mg/kg)	200 ± 6[b]	5/10[b]	50
SAO + sulfatide (10 mg/kg)	237 ± 7[c]	9/10[c]	90

Each point represents the mean ± S.E.M. from 10 rats. [a]$P < 0.001$ vs. sham; [b]$P < 0.05$ vs. SAO + vehicle; [c]$P < 0.001$ vs. SAO + vehicle.

Table 2
Effect of sulfatide on serum and macrophage tumor necrosis factor (TNF-α) in splanchnic artery occlusion (SAO) shocked rats

Treatment	Serum TNF-α (U/ml)	Macrophage TNF-α (U/ml)
Sham + vehicle (1 ml/kg)	N.D.	N.D.
Sham + sulfatide (10 mg/kg)	N.D.	N.D.
SAO + vehicle (1 ml/kg)	398 ± 18[a]	223 ± 19[a]
SAO + sulfatide (10 mg/kg)	37 ± 11[b]	21 ± 8[b]

Serum and macrophages were collected 70 min following the onset of reperfusion. Each point represents the mean ± S.E.M. from six experiments. [a]$P < 0.001$ vs. sham; [b]$P < 0.001$ vs. SAO + vehicle. N.D. = not detectable.

ond layer reagents were then applied, followed by avidin–biotin–horse radish peroxidase complex as a chromogenic substrate, as previously reported (Hsu et al., 1981). The experiments were carried out by two observers (PC, GF) who were unaware of the experimental protocol. The microscope image was sent to a computer-assisted image analyser that analysed the changes in staining. Densitometric analysis of the captured image was performed on a PC computer using image analysis software.

2.7. Drugs

Acetylcholine chloride, phenylephrine hydrochloride, indomethacin and sulfatide were obtained from Sigma. Specific mouse P-selectin antibodies (cross-react with rat P-selectin) were obtained from Pharmigen.

2.8. Statistical analysis

Data are expressed as means ± S.E.M. and were analysed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as criterion for statistical significance. For survival data, statistical analysis was done with Fisher's exact probability test.

3. Results

3.1. Survival

Table 1 summarizes survival rate, percentage survival and survival time for the groups of rats subjected to splanchnic ischaemia-reperfusion injury or sham ischaemia. All sham rats survived the entire 4-h observation period. In contrast, in rats treated with the vehicle, occlusion and reperfusion of the splanchnic region produced a profound shock characterized by a high lethality: no rat survived after 2 h of reperfusion (survival = 80 ± 10 min). Sulfatide increased in a dose-dependent manner the survival rate and time in splanchnic artery occlusion shocked rats. The most effective dose was 10 mg/kg and therefore we used it in the further studies. Furthermore, rats surviving at 4 h were still alive 24 h after the surgical procedures. Administration of P-selectin mAb also resulted in significant protection against splanchnic artery occlusion shock (Table 1).

3.2. Serum and macrophage TNF-α

Serum and macrophage levels of TNF-α were undetectable in sham-operated rats treated either with vehicle or sulfatide. TNF-α was significantly increased in both serum and macrophages collected from splanchnic artery occlusion shocked rats at the end of the reperfusion period (Table 2). The administration of sulfatide significantly blunted the macrophage and serum levels of this cytokine.

3.3. Leucocyte infiltration

Leucocyte infiltration was determined by measurement of myeloperoxidase activity in rats at different times: 0 min before occlusion (basal; at the beginning of the experiment) and 70 min following the onset of reperfusion. Myeloperoxidase levels were significantly increased in the ileum (8.2 ± 0.8 U/g tissue) at 70 min after reperfusion (Table 3) in splanchnic artery occlusion-shocked rats treated with the vehicle.

Administration of sulfatide (10 mg/kg, i.v.) 5 min after occlusion of the splanchnic arteries significantly lowered the ileal increase (1.2 ± 0.4 U/g tissue) in myeloperoxidase activity (Table 3).

Table 3
Effect of sulfatide on myeloperoxidase (MPO) activity of ileum and on white blood cell count of rats subjected to splanchnic artery occlusion (SAO) shock

Treatment	MPO activity in the ileum (U/g tissue)	
	Basal (0 min)	Reperfusion (70 min)
Sham + vehicle (1 ml/kg)	0.1 ± 0.02	0.2 ± 0.03
Sham + sulfatide (10 mg/kg)	0.09 ± 0.01	0.06 ± 0.01
SAO + vehicle (1 ml/kg)	0.1 ± 0.04	8.2 ± 0.8[a]
SAO + sulfatide (10 mg/kg)	0.08 ± 0.08	1.2 ± 0.4[b]
Treatment	White blood count (cell × 10^3/mm^3)	
	Basal (0 min)	Reperfusion (70 min)
Sham + vehicle (1 ml/kg)	12.6 ± 2.1	11.4 ± 1.9
Sham + sulfatide (10 mg/kg)	11.9 ± 1.6	13.1 ± 2.2
SAO + vehicle (1 ml/kg)	12.8 ± 1.3	5.4 ± 2.7[a]
SAO + sulfatide (10 mg/kg)	11.2 ± 3.1	10.5 ± 1.8[b]

Each point represents the mean ± S.E.M. from six experiments. [a]$P < 0.001$ vs. sham; [b]$P < 0.001$ vs. SAO + vehicle.

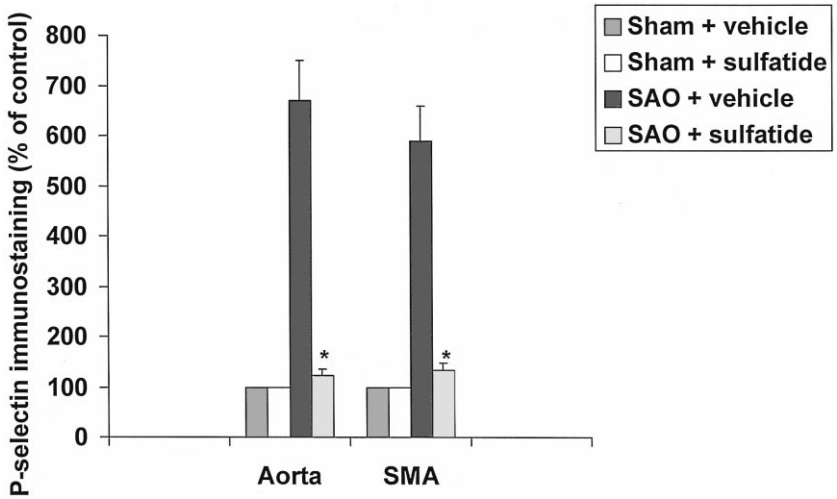

Fig. 1. Effects of vehicle (1 ml/kg, i.v., 5 min after the occlusion of the splanchnic arteries) or sulfatide (10 mg/kg, i.v., 5 min after the occlusion of the splanchnic arteries) on immunohistochemical staining for P-selectin in aortic (Aorta) and superior mesenteric artery (SMA) endothelium from rats subjected to splanchnic ischaemia-reperfusion injury (SAO). Each point represents the mean ± S.E.M. of six experiments. * $P < 0.01$ vs. SAO + vehicle.

3.4. Leucocyte count

The administration of vehicle did not modify the white blood cell count in sham-operated rats (Table 3). In contrast, splanchnic ischaemia-reperfusion injury produced a marked leucopenia. Leucocyte count was markedly decreased at the end of reperfusion (70 min). The administration of sulfatide significantly ameliorated this leucopenia (Table 3).

3.5. P-selectin staining on vascular endothelium

The presence of P-selectin was studied in thoracic aortae and in superior mesenteric arteries collected 70 min following release of the occlusion. Immunohistochemical evaluation indicated that a very low constitutive staining of P-selectin was present in sham-operated animals. By contrast, samples obtained from splanchnic artery occlusion-shocked rats had increased levels of P-selectin staining. Aortic and mesenteric endothelium obtained from splanchnic artery occlusion shocked rats treated with sulfatide showed a marked reduction in P-selectin immunostaining. (Fig. 1).

3.6. Vascular reactivity of aortic rings

Addition of phenylephrine (100 nM) to the organ bath contracted intact aortic rings (80–90% of the maximum response). These rings were relaxed in a concentration-dependent manner by acetylcholine (10 nM–10 μM). The relaxant effect of acetylcholine was significantly smaller in aortic rings obtained from splanchnic artery occlusion-shocked rats than from sham-operated rats (Fig. 2). Administration of sulfatide significantly improved the responsiveness to acetylcholine of aortic rings obtained from shocked rats (Fig. 2).

In intact aortic rings prepared from splanchnic artery occlusion-shocked rats, the contractile response to phenyl-

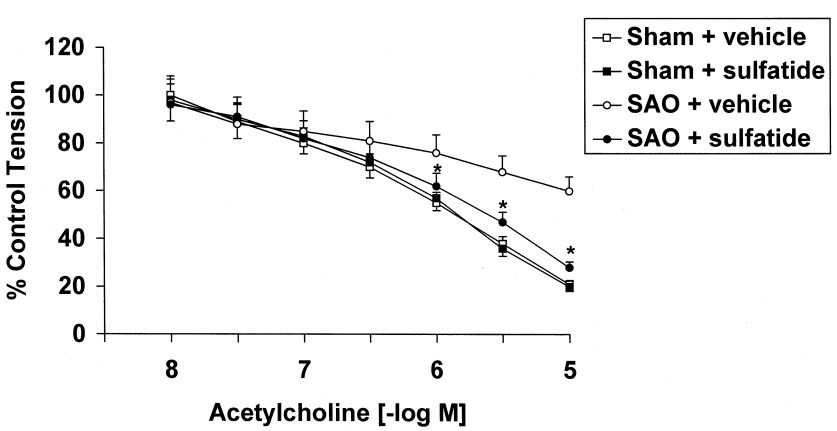

Fig. 2. Relaxant effects of acetylcholine (ACh) in aortic rings (contracted with phenylephrine, 100 nM) of sham operated rats and rats subjected to splanchnic ischaemia-reperfusion injury (SAO) treated with vehicle (1 ml/kg, i.v., 5 min after the occlusion of the splanchnic arteries) or sulfatide (10 mg/kg, i.v., 5 min after occlusion of the splanchnic arteries). Each point represents the mean ± S.E.M. of six experiments. * $P < 0.01$ vs. SAO + vehicle.

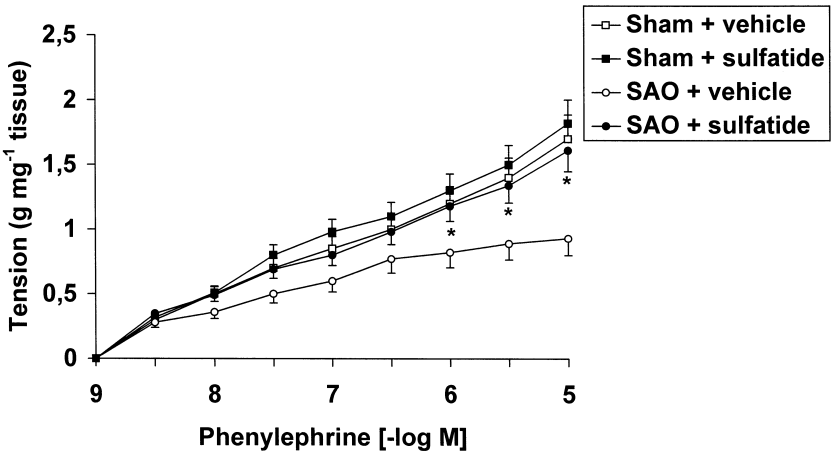

Fig. 3. Contractile response to cumulative doses of phenylephrine (PE) in endothelium-denuded aortic rings from sham-operated rats and rats subjected to splanchnic ischaemia-reperfusion injury (SAO) treated with vehicle (1 ml/kg, i.v., 5 min following the occlusion of the splanchnic arteries) or sulfatide (10 mg/kg, i.v., 5 min after the occlusion of the splanchnic arteries). Each point represents the mean ± S.E.M. of six experiments. * $P < 0.01$ vs. SAO + vehicle.

ephrine (1 nM–10 μM) was significantly reduced. The maximum force of contraction induced by 10 μM phenylephrine in aortic rings from sham-operated rats was 1.8 ± 0.3 g/mg tissue, whereas it was 0.9 ± 0.1 g/mg in rings from splanchnic artery occlusion-shocked rats. Removal of the endothelium did not increase the constrictor response elicited by phenylephrine in rat aortic rings obtained from both splanchnic artery occlusion-shocked rats and sham operated animals (Fig. 3). However, the contractile response to phenylephrine in endothelium-denuded aortic rings was significantly smaller in splanchnic artery occlusion-shocked rats than in sham-operated animals. Administration of sulfatide improved the impaired contractile response to phenylephrine in splanchnic artery occlusion-shocked rats (Fig. 3).

3.7. Mean arterial blood pressure

Occlusion of the splanchnic arteries produced a marked increase in mean arterial blood pressure. Subsequently mean arterial blood pressure decreased upon release of the occlusion. The administration of sulfatide significantly blunted the reduction in mean arterial blood pressure (Table 4).

Table 4
Effect of sulfatide on mean arterial blood pressure (mm Hg) in splanchnic artery occlusion (SAO shocked rats)

Treatment	Mean arterial blood pressure (mm Hg)		
	Basal	End of occlusion	End of reperfusion
Sham + vehicle (1 ml/kg)	95 ± 6	92 ± 5	93 ± 9
Sham + sulfatide (10 mg/kg)	92 ± 5	94 ± 4	91 ± 7
SAO + vehicle (1 ml/kg)	93 ± 5	131 ± 6[a]	21 ± 3[a]
SAO + sulfatide (10 mg/kg)	91 ± 4	127 ± 7[b]	88 ± 7[b]

Each point represents the mean ± S.E.M. from 10 rats. [a] $P < 0.001$ vs. sham; [b] $P < 0.01$ vs. SAO + vehicle. Mean arterial blood pressure was measured at several time points: 0 min before occlusion (basal), 45 min after occlusion of the splanchnic arteries (end of occlusion) and 70 min following the onset of reperfusion (end of reperfusion).

4. Discussion

Adhesion of polymorphonuclear leucocytes to endothelial cells is one of the important steps involved in the development of splanchnic artery occlusion shock (Altavilla et al., 1995). The adhesion of neutrophils occurs in multiple steps facilitated by several species of adhesion molecules present on polymorphonuclear leucocytes and endothelial cells (Granger and Kubes, 1994). In the inflammatory response, neutrophils initially interact with endothelial cells via selectins (E-, P- and L-selectins), which are responsible for tethering polymorphonuclear leucocytes (Tedder et al., 1995). The role of selectins in tethering has been shown in vivo in selectin-deficient mice. After surgical stimulus, leucocyte rolling along the endothelial cells was significantly impaired in P-selectin and L-selectin-deficient mice (Ley et al., 1995).

After the tethering stage polymorphonuclear leucocytes adhere firmly to endothelial cells by interactions between membrane integrins and immunoglobulin superfamilies, causing neutrophils to migrate to inflammatory sites (Granger and Kubes, 1994). Adhered or migrated polymorphonuclear leucocytes undergo activation and release several toxic substance including reactive oxygen species and proteolytic enzymes that causes tissue injury (Lucchesi, 1991; Granger and Kubes, 1994). The availability of sulfatide, sulfated galactosylceramide, a substance capable of blocking P-selectin (Needham and Schnaar, 1993; Todderud et al., 1997) prompted us to investigate the effect of complete selectin antagonism in splanchnic artery occlusion shock.

Our data suggest that sulfatide significantly increased the resistance of rats to the experimental procedure of splanchnic artery occlusion shock: indeed complete blockade of P-selectin succeeded in significantly improving both survival rate and survival time. These results confirm the potential anti-inflammatory effects of sulfatide (Ding et al., 1997; Higashi et al., 1997). Shocked rats had also leucopenia and marked leucocyte infiltration, as shown by the increase in myeloperoxidase in the ileum. This increase in neutrophil adhesion was also accompanied by a marked increase in P-selectin staining in the aorta and in the superior mesenteric artery, thus suggesting that P-selectin is involved, at least in part, in the pathogenesis of splanchnic artery occlusion shock. The results showing that treatment with a P-selectin mAb improved the survival rate and time of rats subjected to the experimental procedure of splanchnic artery occlusion shock strongly support this hypothesis.

It has also been suggested that the pleiotropic cytokine TNF-α plays an important role in the pathogenesis of ischaemic states (Squadrito et al., 1993). In fact, TNF-α may induce the expression of several adhesion molecules, including selectins, and cause leucocytes to adhere (Mantovani and Dejana, 1989) to the vascular endothelium where they secrete deleterious mediators (i.e., oxygen free radicals, leukotrienes, cytokines, etc.) able to amplify the vascular damage. In agreement with these earlier data, rats subjected to the experimental procedure of splanchnic artery occlusion shock had enhanced serum and macrophage levels of TNF-α. These data, taken together, suggest that TNF-α may induce the expression of selectin in vivo during splanchnic artery occlusion shock.

Surprisingly, sulfatide treatment decreased the serum and macrophage levels of the pleiotropic cytokine. This effect could be due to reduced macrophage recruitment and activation as a result of the selectin blockade. Furthermore the reduction in TNF-α following sulfatide treatment also explains why the drug blunted the expression of P-selectin in the vascular endothelium.

As far as vascular dysfunction is concerned, it has been suggested that TNF-α may impair the release of nitric oxide (NO) from endothelial cells (Aoki et al., 1990), thus leading to a reduced production of endothelial-derived relaxing factor. However, administration of recombinant human TNF-α to conscious rats has been reported to induce a decrease in mean arterial blood pressure and to produce vascular hyporeactivity to contractile agents, an effect that is reversed by inhibitors of NO synthesis (Takahashi et al., 1992). This phenomenon is likely due to TNF-α-induced stimulation of the Ca^{2+}-independent NO synthase in vascular smooth muscle (Busse and Mulsch, 1990).

Aortic rings from rats subjected to splanchnic-ischaemia reperfusion injury had a markedly reduced responsiveness to the vasorelaxant effects of acetylcholine; this finding indicates a reduced production of NO in this type of experimental ischaemia-reperfusion injury. However our results also showed that there was a reduced vascular sensitivity to vasoconstrictor stimuli. This impaired vascular reactivity, as suggested for other models of experimental shock (Thiemermann et al., 1993), is a consequence of an overproduction of NO by the inducible NO synthase (Squadrito et al., 1994a). Therefore all these data, taken together, suggest that in splanchnic artery occlusion shock (i) NO generated by the endothelial NO synthase is blunted, while (ii) NO produced by the inducible NO synthase is increased. These opposite effects in splanchnic artery occlusion shock are induced by TNF-α (Squadrito et al., 1994a); this inflammatory cytokine in fact either inhibits endothelial NO synthase and stimulates inducible NO synthase. This hypothesis is confirmed by the evidence that an inhibitor of TNF-α synthesis is able to reverse this complex vascular dysfunction (Squadrito et al., 1994b). In the present paper aortic rings collected from rats subjected to ischaemia-reperfusion injury and treated with sulfatide exhibited a greater contractile response to phenylephrine and improved responsiveness to acetylcholine when compared to those from vehicle-treated rats. Thus it can be hypothesized that sulfatide improves the vasoconstrictor response to phenylephrine and the relaxant effect of acetylcholine by inhibiting the detrimental vascular effects of TNF-α. Indeed, the impairment of acetylcholine responses might also be the consequence of a leucocyte–endothelial interaction. In fact adherence of leucocytes to the endothelium may, by releasing oxygen-derived free radicals, impair the release of NO from endothelial cells. Therefore therapeutic approaches that reduce leucocyte adherence to the endothelium are expected to restore the impairment in NO dysfunction. This hypothesis led us to conclude that sulfatide may also improve responsiveness to acetylcholine by reducing leucocyte–endothelial interaction.

In conclusion, we have shown that sulfatide inhibits in 'vivo' TNF-α: the sulfatide-induced inhibition of this inflammatory cytokine, at least in splanchnic artery occlusion shock, increases survival, reduces P-selectin expression and leucocyte infiltration in the ileum and reverses vascular failure. These findings would suggest that sulfatide may represent a new therapeutic approach to the treatment of circulatory shock.

Acknowledgements

This project was supported in part by a grant from CNR Italy (95.02181.CT04).

References

Altavilla, D., Berlinghieri, M.C., Seminara, S., Iannello, D., Foca, A., Mastroeni, P., 1989. Different effects of bacterial lipopolysaccharide on superoxide anion production by macrophages from normal and tumor bearing rats. Immunopharmacology 17, 99–105.

Altavilla, D., Squadrito, F., Canale, P., Ioculano, M., Campo, G.M., Squadrito, G., Ammendolia, L., Caputi, A.P., 1995. E-selectin in-

volvement in the pathogenesis of splanchnic artery occlusion shock. Eur. J. Pharmacol. 272, 223–229.

Aoki, N., Johson, G. III, Lefer, A.M., 1990. Beneficial effects of two forms of NO administration in feline splanchnic artery occlusion shock. Am. J. Physiol. 321, G275–G281.

Arfors, K.E., Lundberg, C., Lindbom, L., Lundberg, K., Beatty, P.G., Harlan, J.M., 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. Blood 69, 338–340.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S., Gimbrone, M.A., 1987. Identification of an inducible endothelial–leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA 84, 9238–9242.

Busse, R., Mulsch, A., 1990. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Lett. 275, 87–90.

Canale, P., Squadrito, F., Zingarelli, B., Altavilla, D., Ioculano, M., Campo, G.M., Caputi, A.P., 1993. Splanchnic artery occlusion shock: vinblastine induced leukopenia reduces tumor necrosis factor and thromboxane A_2 formation and increases survival rate. Pharmacol. Res. 27, 61–71.

Caputi, A.P., Rossi, F., Carney, K., Brezenoff, H.E., 1980. Modulatory effect of brain acetylcholine on reflex-induced bradycardia and tachycardia in conscious rats. J. Pharmacol. Exp. Ther. 215, 309–316.

Ding, Z., Kawashima, H., Suzuki, T., Ward, P.A., Miyasaka, M., 1997. A sulfatide receptor distinct from L-selectin is involved in lymphocyte activation. FEBS Lett. 418, 310–314.

Granger, D.N., Kubes, P., 1994. The microcirculation and inflammation: modulation of leukocyte–endothelial cell adhesion. J. Leukoc. Biol. 55, 662–668.

Higashi, H., Suzuki, Y., Mukaida, N., Takahash, N., Niyamoto, D., Matsushima, K., 1997. Intervention in endotoxin shock by sulfatide (I3SO3-GalCer) with a concomitant reduction in tumor necrosis alpha production. Infect. Immun. 65, 1223–1227.

Hsu, S.M., Raine, L., Fanger, H., 1981. A comparative study of the peroxidase–antiperoxidase method and an avidin–biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. 75, 734–737.

Key, K., Gaehtgens, P., Fennie, C., Singer, M.S., Lasky, L.A., Rosen, S.D., 1991. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vitro. Blood 77, 2553–2555.

Ley, K., Bullard, D.C., Arbones, M.L., Bosse, R., Vestweeber, D., Tedder, T.F., Beaudet, A.L., 1995. Sequential contribution of L- and P-selectins to leukocyte rolling in vivo. J. Exp. Med. 181, 669–675.

Lucchesi, B.R., 1991. Modulation of leukocyte-mediated myocardial reperfusion injury. Annu. Rev. Physiol. 84, 400–421.

Mantovani, A., Dejana, E., 1989. Cytokines as communication signals between leukocytes and endothelial cells. Immunol. Today 10, 370–375.

Mullane, K.M., Kraemer, M.R., Smith, B., 1985. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. J. Pharmacol. Methods 14, 156–157.

Mulligan, M.S., Johnson, K.J., Todd, R.F. III et al., 1993. Requirements for leukocyte adhesion molecules in nephrotoxic nephritis. J. Clin. Invest. 91, 577–587.

Needham, L.K., Schnaar, R.L., 1993. The HNK-1 reactive sulgoglucuronyl glycolipids are ligand for L-selectin and P-selectin but not E-selectin. Proc. Natl. Acad. Sci. USA 90, 1359–1363.

Patel, K.D., Zimmerman, G.A., Prescott, S.M., Mcever, R.P., Mcintyre, T.M., 1991. Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J. Cell Biol. 112, 749–759.

Romson, J.L., Hook, B.G., Kunkel, S.L., Abrams, G.D., Schork, M.A., Lucchesi, B.R., 1983. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 67, 1016–1021.

Ruff, M.R., Gifford, G.E., 1980. Purification and physicochemical characterization of rabbit tumor necrosis factor. J. Immunol. 125, 1671–1675.

Simpson, P.J., Todd, R.F. III, Fantone, J.C., Mickelson, J.K., Griffin, J.D., Lucchesi, B.R., 1988. Reduction of experimental canine myocardial reperfusion injury by monoclonal antibody (anti Mo1, anti-CD11b) that inhibits leukocyte adhesion. J. Clin. Invest. 81, 624–630.

Smith, C.W., Marlin, S.D., Rothlein, R., Toman, C., Anderson, D.C., 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule 1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. J. Clin. Invest. 83, 2008–2017.

Squadrito, F., Altavilla, D., Ioculano, M., Calapai, G., Zingarelli, B., Saitta, A., Campo, G.M., Rizzo, A., Caputi, A.P., 1992. Passive immunization with antibodies against tumor necrosis factor TNF-α protects from the lethality of splanchnic artery occlusion shock. Circ. Shock 37, 236–244.

Squadrito, F., Altavilla, D., Zingarelli, B., Ioculano, M., Calapai, G., Campo, G.M., Caputi, A.P., 1993. Tumor necrosis factor involvement in myocardial ischaemia-reperfusion injury. Eur. J. Pharmacol. 273, 223–230.

Squadrito, F., Altavilla, D., Canale, P., Ioculano, M., Campo, G.M., Ammendolia, L., Ferlito, M., Zingarelli, B., Squadrito, G., Saitta, A., Caputi, A.P., 1994a. Participation of tumor necrosis factor and nitric oxide in the mediation of vascular dysfunction in splanchnic artery occlusion shock. Br. J. Pharmacol. 113, 1153–1158.

Squadrito, F., Altavilla, D., Canale, P., Ioculano, M., Campo, G.M., Ammendolia, L., Squadrito, G., Saitta, A., Calapai, G., Caputi, A.P., 1994b. Contribution of intercellular adhesion molecule 1 (ICAM-1) to the pathogenesis of splanchnic artery occlusion shock in the rat. Br. J. Pharmacol. 113, 912–916.

Takahashi, K., Ando, K., Ano, A., Shimosawa, T., Ogata, E., Fujita, T., 1992. Tumor necrosis factor-α induces vascular hyporesponsiveness in Sprague–Dawley rats. Life Sci. 50, 1437–1444.

Tedder, T.F., Steeber, D.A., Chen, A., Engel, P., 1995. The selectins: vascular adhesion molecules. FASEB J. 9, 866–871.

Thiemermann, C., Szabo, C., Mitchell, J.A., Vane, J.R., 1993. Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide. Proc. Natl. Acad. Sci. USA 90, 267–271.

Todderud, G., Nair, X., Lee, D., Alford, J., Davern, L., Stanley, P., Bachand, C., Lapointe, P., Marinier, A., Martel, A., Menard, M., Wright, J.J., Bajorath, J., Hollenbaugh, D., Aruffo, A., Tramposch, K.M., 1997. BMS-190394, a selectin inhibitor, prevents rat cutaneous inflammatory reactions. J. Pharmacol. Exp. Ther. 282, 1298–1304.

Welbourn, R., Goldman, G., Kobzik, L., Paterson, I.S., Valeri, C.R., Shepro, D., Hechtman, H.B., 1992. Role of neutrophil adherence receptors (CD18) in lung permeability following lower torso ischemia. Circ. Res. 71, 82–86.